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# Assessing pyrethroid resistance status in the <i>Culex pipiens<i> complex (Diptera: Culicidae) from the northwest suburbs of Chicago, Illinois using Cox regression of bottle bioassays and other detection tools

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| Abstract: | Culex pipiens  complex is an important vector of epizootic and zoonotic pathogens, including West Nile virus. Chicago, Illinois and its suburbs have suffered high incidence of human West Nile virus infections in the past. This makes abatement programs in and around the Chicago area an essential service. The control of  Cx. pipiens  is often complicated by rapidly evolving resistance to pyrethroids, which are the most widely used chemical class in US mosquito abatement programs. The present study assessed Sumithrin® resistance in  Cx. pipiens  collected from five locations around Cook County, Illinois, neighboring the city limits of Chicago. According to CDC guidelines, samples from all five locations demonstrated some resistance to Sumithrin®. When assessed with Anvil®, a formulated product made of Sumithrin® synergized with piperonyl butoxide, susceptibility was rescued in mosquitoes from three out of the five locations, suggesting involvement of mixed-function oxidases and/or carboxylesterases in Sumithrin® resistance at these locations. Not all locations had susceptibility rescued by Anvil®, but these locations had relatively low knockdown resistance allele frequencies, suggesting that mechanisms other than knockdown resistance may be involved. Enzyme activities did not reveal any marked trends that could be related back to mortality in the bottle bioassays, which highlights the need for multiple types of assays to infer enzymatic involvement in resistance. Future directions in pyrethroid resistance management in Chicago area  Cx. pipiens  are discussed. |
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4       Full title:  Assessing pyrethroid resistance status in the *Culex pipiens* complex

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6                 regression of bottle bioassays and other detection tools

7

8           Short title: Assessing pyrethroid resistance in Chicago *Culex pipiens*

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29

# Abstract

*Culex pipiens* complex is an important vector of epizootic and zoonotic pathogens, including West Nile virus. Chicago, Illinois and its suburbs have suffered high incidence of human West Nile virus infections in the past. This makes abatement programs in and around the Chicago area an essential service. The control of *Cx. pipiens* is often complicated by rapidly evolving resistance to pyrethroids, which are the most widely used chemical class in US mosquito abatement programs. The present study assessed Sumithrin® resistance in *Cx. pipiens* collected from five locations around Cook County, Illinois, neighboring the city limits of Chicago. According to CDC guidelines, samples from all five locations demonstrated some resistance to Sumithrin®. When assessed with Anvil®, a formulated product made of Sumithrin® synergized with piperonyl butoxide, susceptibility was rescued in mosquitoes from three out of the five locations, suggesting involvement of mixed-function oxidases and/or carboxylesterases in Sumithrin® resistance at these locations. Not all locations had susceptibility rescued by Anvil®, but these locations had relatively low knockdown resistance allele frequencies, suggesting that mechanisms other than knockdown resistance may be involved. Enzyme activities did not reveal any marked trends that could be related back to mortality in the bottle bioassays, which highlights the need for multiple types of assays to infer enzymatic involvement in resistance. Future directions in pyrethroid resistance management in Chicago area *Cx. pipiens* are discussed.

# Introduction

The *Culex pipiens* complex (*Cx. pipiens*) plays a major role in vectoring several epizootic and zoonotic pathogens significant to birds and humans, including West Nile virus (WNV) and St.

52   Louis encephalitis [1, 2]. WNV is considered the most widespread arbovirus in the United States

53   [3]. In the summer of 2002, cases of WNV in Chicago surged, with 884 cases and 66 deaths,

54   followed by another outbreak in 2003 but with only 53 cases. Chicago resides in Cook

55   County, Illinois, and is home to an estimated 5.1 million people. Factors related to geography,

56   housing, population, and abatement strategies around Chicago and the surrounding county makes

57   this a potential high-risk region for WNV cases [5].

58       Typically thought to feed primarily on birds, *Cx. pipiens* from the upper Midwest are

59   aggressively anthropophagic [6]. The *Cx. pipiens* complex is currently comprised of *Cx.*

60   *australicus*, *Cx. pipiens*, and *Cx. quinquefasciatus*, with various hybridizations and biotypes

61   among the species, two of which are known as form *pallens* and *molestus* [7]. Form *molestus* is

62   present in Chicago [8] but does not appear to be significantly introgressed into these *Cx. pipiens*

63   populations [9]. The *molestus* form is important because it appears to be more strongly

64   associated with anthropophagy, increasing its potential to transmit pathogens such as WNV [10,

65   11]. Mosquitoes from the *Cx. pipiens* complex are primarily active at night, which makes them

66   particularly good targets for nightly insecticidal control efforts using ultra-low volume (ULV)

67   fogging [12, 13]. ULV fogging remains the most competent tool available for quickly reducing

68   WNV and other mosquito vector populations during times of arboviral disease outbreaks [14].

69       ULV products typically contain one of two classes of insecticides, either pyrethroids or

70   rarely organophosphates. Pyrethroids are generally safer than organophosphates to vertebrates, as

71   they break down into safe metabolites relatively quickly and they are cheap. But resistance to

72   pyrethroids has been well-documented in *Cx. pipiens* throughout the United States (reviewed in

73   [15]). Among the likeliest of the proposed physiological mechanisms that confer this resistance

74   are target site mutations in the para region of the voltage-sensitive sodium channel and metabolic

75    detoxification by a few families of enzymes, including mixed-function oxidases (MFO) known

76    as cytochrome P450s, carboxylesterases (CarE), and glutathione *S*-transferases (GST). How

77    these mechanisms combine to confer resistance is not well-understood but nevertheless should be

78    monitored by mosquito control operations to better understand their relationship to product

79    failure.

80          In the present study, CDC bottle bioassays were conducted as an initial screening for

81    resistance to Sumithrin® (d-phenothrin), a Type I pyrethroid and Anvil® (Sumithrin® plus

82    piperonyl butoxide, a synergist) in *Cx. pipiens* from four sites in the northwest suburbs of Cook

83    County, Illinois. These sites were in Wheeling, Arlington Heights, and two sites in Des Plaines.

84    As a post hoc laboratory analysis, organisms from the bottle bioassays were genotyped for single

85    nucleotide polymorphisms in the voltage-sensitive sodium channel that confers knockdown

86    resistance (*kdr*). Mosquitoes from these samples also were tested for enzymatic activity. Finally,

87    bottle bioassay data was analyzed using clustered Cox regression using time-dependent

88    covariates to account for non-proportional hazards when they occurred [16]. This approach to

89    bottle bioassay data analysis provides additional information on the rate of mortality between

90    start and endpoint. Using Cox regression in this way also is beneficial because non-proportional

91    hazards due to heterogeneity of resistance factors in recently field-derived strains can be

92    accounted for in this type of model.

93

## 94    Methods

## 95    Mosquito sources and pyrethroid exposure histories

96        Five study sites were selected within the Northwest Mosquito Abatement District, located

97    in Cook County, IL, USA (Fig 1, Table 1). Each site is approximately 2.59 km$^2$ and located 0.8

98    km to 8 km from other sites. Within each site, four homeowners allowed mosquito collections

99    with one CDC gravid trap (John W. Hock Company, Gainesville, FL). Gravid traps were baited

100    with an infusion of 75 g Timothy Complete rabbit food (Kaytee Products Inc., Chilton, WI), 58 g

101    Kentucky bluegrass (*Poa pratensis*) collected from the district's property, 2.5 g lactalbumin

102    whey protein (TGS Nutrition, Las Vegas, NV), 0.22 g Altosid® pellets (Central Life Sciences,

103    Schaumburg, IL), and 9.46 L water. Gravid infusion was stored outside for at least one week

104    before use. *Cx. pipiens* egg rafts were collected from gravid trap basins every other week

105    between the months of July and August in 2020. To ensure the correct species was collected, egg

106    rafts were hatched and reared individually until species could be identified at second instar [17].

107    Mosquitoes from the same site and collection day were pooled together and reared at 27°C and in

108    a 16:8 hour light:dark cycle. Larvae were fed with ground TetraMin® tropical fish flakes

109    (Spectrum Pet Brands LLC, Blacksburg, VA) and adults were fed a 10% sucrose solution.

110

111    **Fig 1. Trapping locations of the seven strains tested.** A = WHE (Wheeling), B = AHB

112    (Arlington Heights South Catch Basin), C = DPN (Des Plaines North), D = DPS and DPSB (Des

113    Plaines South and Des Plaines South Catch Basin).

114

115    **Table 1. Sampling collection and analysis information.**

| Strain Location (Latitude, Longitude) | Abbreviation | Date of Bottle Bioassay (Insecticide Used) | Post Hoc Assays$^2$ |
|---|---|---|---|

| Location | Code | Date (Product) | Assay |
|---|---|---|---|
| Arlington Heights catch Basin (42.054977, – 87.984398) | AHB | August 5 (Sumithrin®) | Genotype, Enzyme |
| | | August 7 (Anvil®) | None |
| Wheeling (42.117205, – 87.936830) | WHE | August 11 (Sumithrin®) | Genotype, Enzyme |
| | | August 13 (AnvilTM) | None |
| | | August 14 (Anvil®) | None |
| Des Plaines South (42.029653, – 87.930887) | DPS | August 26 (Sumithrin®) | Genotype, Enzyme |
| Des Plaines South catch basin (42.024806, – 87.925787) | DPSB | August 19 (Sumithrin®) | Genotype |
| | | August 21 (AnvilTM, Anvil®) | None |
| Des Plaines North (42.059309, – 87.912542) | DPN[1] | August 5 (Sumithrin®) | None |
| | | August 7 (Anvil®) | Genotype |
| Des Plaines North, first resample | DPN1[1] | August 19 (Sumithrin®) | Enzyme |

| | | August 21 (AnvilTM, Anvil®) | None |
| --- | --- | --- | --- |
| Des Plaines North, second resample | DPN2[1] | September 1 (Sumithrin®) | Enzyme |

[1] DPN, DPN1, and DPN2 represent different generations of mosquitoes reared from the same sight. DPN2 was collected after DPN1, and DPN1 was collected after DPN.

[2] Genotyping was for the L982F *kdr* mutation (Genotype) using mosquitoes from the treatment bottles, and enzyme quantity and activities tested were mixed-function oxidase, α- and β-carboxylesterase, and glutathione *S*-transferase enzyme activity (Enzyme) using non-treated control mosquitoes run alongside the treated bottles during the bottle bioassays.

Sprays were conducted once a week for 5 weeks in Wheeling, Arlington Heights North, and Des Plaines South in 2019 and 2020, starting in July and ending in August. In 2019, Zenivex® E20 (20% etofenprox) in a 1:1 mix with mineral oil (10% etofenprox) was used and in 2020, Anvil® 10+10 was sprayed at 0.0036 Lb per acre of active ingredient and piperonyl butoxide (PBO). From 2013 – 2018 Northwest Mosquito Abatement District averaged 1 spray event (etofenprox) per year in these areas. The sites where the mosquitoes were obtained are in residential neighborhoods with numerous parks, schools, and manicured lawns. Numerous residential mosquito/pest control companies operate in these residential areas. Pyrethroids are commonly used by homeowners, park districts, schools, and golf courses [18].

**Bottle bioassays**

134     CDC bottle bioassays were completed using three different insecticide solutions on adult

135     *Cx. pipiens* aged 3-6 days [19] (Table 1). Most bottle bioassays were completed with technical

136     grade Sumithrin® (provided in the CDC bottle bioassay kit) diluted with acetone to 20 µg/bottle,

137     the diagnostic dose used in the bottle bioassay guidelines. The diagnostic time for Sumithrin® in

138     *Cx. pipiens* adults is 30 minutes. This time and concentration represent empirically determined

139     parameters that are specific for this species to display 100% mortality when fully susceptible

140     ([19], section 3.2). The second solution consisted of Anvil® 10+10 (Clarke® Mosquito Control

141     Products, Inc., Roselle, IL) diluted to 22.2 µg Sumithrin® and 22.2 µg PBO per bottle [20]. This

142     dose of Sumithrin® is similar to the diagnostic dose in the guidelines. The second Anvil® 10+10

143     solution was made with the same protocol, instead using the 1:1 mineral oil tank mix

144     (abbreviated AnvilTM) diluted with acetone to 11.1 µg Sumithrin® and 11.1 µg PBO per bottle.

145     250 mL glass Wheaton bottles were treated with 1 mL insecticide solution according to the CDC

146     procedure and allowed to dry for at least four hours. Insecticide solutions were stored at 4 °C.

147     Control bottles were treated with 1 mL acetone and allowed to dry for four hours. 15-25 mixed

148     sex *Cx. pipiens* were aspirated into each bottle and knockdown was recorded every 5 min for 45

149     min, then every 15 min until 120 min total. At the completion of the bioassay, mosquitoes were

150     killed and stored at –80 °C.

151

## Genotyping for knockdown resistance alleles

153     Genotyping for the 982L and 982F alleles, canonically known as 1014L and 1014F

154     alleles, was conducted using a PCR-based melt curve assay modelled on the assay of Saavedra-

155     Rodriquez et al. [21] but with primers designed for the *Cx. pipiens* complex (Table 2). Controls

156     for the LL, FF, and the LF genotypes, from mosquitoes that had been previously genotyped by

157    Sanger sequencing, were included to ensure that the assay reliably detected all three genotypes

158    common to the US. No template (negative) controls were also included. Individual organisms

159    collected from the CDC bottle bioassays were loaded into 96-well plates (Omni International,

160    Kennesaw, GA) with 400 μL of nuclease free water and cubic zirconium beads (BioSpec

161    Products, Bartlesville, OK). Plates were sealed with Teflon™ sealing mats and homogenized for

162    60 seconds at 30 hertz (Omni International, Kennesaw, GA). Samples were centrifuged for 1 min

163    at 805g and then maintained on ice until assay setup. PCR master mix was prepared in sufficient

164    quantity for 400 10 μL reactions (2,000 μL SYBR Select, 1,161.2 μL nuclease free water, 2.4 μL

165    of Cxq_1014L primer, 13.2 μL of Cxq_1014F primer, 10.0 μL of Cxq_1014S primer, and 13.2

166    μL of Cxq_1014_3' primer). Eight microliters of mastermix was added to each well of a 384-

167    well plate (Thermo Fisher Scientific, Waltham, MA) followed by 2 μL of centrifuged

168    homogenate using an epMotion 5075 liquid handling system (Eppendorf, Hamburg, Germany)

169    with filtered tips. Amplification and melt curve data were collected on an Applied Biosystems

170    QS6 (Thermo Fisher Scientific, Waltham, MA) using default fast cycling conditions.

171    Determination of alleles present in a sample was assessed by examination of the derivative melt

172    curve for temperature peaks ($T_m$) as in [21] (Fig 2). In this assay, an LL genotype is

173    characterized by a distinct $T_m$ of 85.3 ± 0.5 °C, an FF by a $T_m$ of 82.6 ± 0.5 °C, and an LF

174    heterozygote has peaks at both $T_m$s (Fig. 2). Samples that did not amplify or that amplified with a

175    cycle threshold greater than 35 were excluded from analysis

176

177    **Table 2. Primers and genomic locations for *Cx. pipiens kdr* 1014 melt curve assay.**

178    <u>Primer</u>          <u>Genomic location</u>                    <u>Sequence</u>

179    Cxq_1014L    NC51862.1: 22646376-22646398

180         **GCGGGCAGGGCGGCGGGGGCGGGG**TTCACGCTGGAATACTCACGACTA

181    Cxq_1014F    NC51862.1: 22646376-22646397

182         **GG**TTCACGCTGGAATACTCACGACA

183    Cxq_1014S    NC51862.1: 22646376-22646398

184         **AGCGCGGAGCGCGG**TTCACGCTGGAATACTCACGACTG

185    Cxq_1014_3'  NC51862.1: 22646459-22646483

186         GGATCGAATCCATGTGGGACTGCAT

187    Primer sequences in bold are tails added to change melting temperatures following the method of

188    [21].

189

190    **Fig 2. *Cx. pipiens kdr* 1014 melt curve assay analysis.** Representative melt curve assay results

191    for a 1014L homozygote (LL), a 1014F homozygote (FF), a 1014LF heterozygote (LF), and a

192    nuclease-free water blank (NFW) showing distinct melting temperatures for the L (85.3 °C) and

193    F (82.6 °C) alleles.

194

## Enzyme activity assays

196        The content of mixed-function oxidases (MFO), and the activity of α- and β-

197    carboxylesterases (α-, β-CarE), and glutathione *S*-transferases (GST) were determined using

198    modified methods from [22] using only freeze-killed control mosquitoes from the bottle

199    bioassays (i.e., mosquitoes that were not exposed to any toxicant). Where multiple bottle

200    bioassays were done on the same strain within a week (i.e., the same generation), enzyme

201    content and activities were assessed on only one set of controls from that week. Because sample

202    size for the controls varied between nine and thirty individuals, eight individual mosquitoes were

203    used from each strain. To produce the enzyme source, mosquitoes were placed into 2.0 mL screw

204    cap microcentrifuge tubes loaded with two 2.0 mm zirconium oxide beads and 400 μL of 100

205    mM sodium phosphate buffer (pH 7.4) with no chelating agents or protease inhibitors.

206    Mosquitoes were homogenized with a bead mill and then centrifuged at 10,000 x g at 4 °C for 10

207    min. The resulting supernatant was used as the enzyme source for all subsequent activity assays

208    as well as for soluble protein determination.

209        The MFO content in samples was determined using the 3,3',5,5'-tetramethylbenzidine

210    (TMB) method originally devised by [23]. This assay does not measure the activity of MFOs

211    toward a model substrate. Instead, it measures the heme content in the sample, the majority of

212    which is thought to belong to MFOs [24]. A 0.2% TMB solution was freshly prepared in

213    methanol and then further diluted to 0.05% in 250 mM sodium acetate buffer (pH 5.0). A 20 μL

214    volume of supernatant was added to a 96-well plate in duplicate followed by 200 μL of 0.05%

215    TMB solution. The reaction was started by adding 25 μL of 3% $H_2O_2$ to each well and then

216    incubating the plate at room temperature for 10 min inside a dark cabinet. Results were

217    compared to a standard curve generated from cytochrome C using the same reagents and

218    volumes as the samples ($R^2 = 0.997$). After the incubation period, wells were read at 620 nm in a

219    BioTek Epoch 2 spectrophotometer (BioTek, Santa Clara, CA). Units of activity were thus

220    reported as cytochrome equivalents.

221        For α- and β-CarE activity, 15 μL of supernatant was incubated with 135 μL of freshly

222    prepared 0.3 mM α- or β-naphthyl acetate (final reaction concentration 0.27 mM in wells) in

223    duplicate in a 96-well plate covered with a lid. Incubation lasted for 15 min at room temperature

224    inside a dark cabinet. The reaction was stopped by adding 50 μL of freshly prepared 0.3% Fast

225    Blue B in 5.0% sodium dodecyl sulphate solution. Color was allowed to develop for 5 min at

226    room temperature in the dark cabinet. The α-naphthyl acetate (α-CarE) samples were read at 600

227    nm and the β-naphthyl acetate (β-CarE) samples were read at 550 nm. Standard curves of α- and

228    β-naphthol were run in triplicate (both $R^2 = 0.999$) and used to quantify the enzymatic

229    conversion of α- and β-naphthyl acetate to α- and β-naphthol, respectively.

230        For GST activity, 20 μL of supernatant was added in duplicate to wells of a 96-well plate.

231    The substrate mixture consisted of 1 mL of 21 mM 1-chloro-2,4-dinitrobenzene (CDNB) in

232    methanol added to 10 mL of freshly prepared 10 mM of reduced glutathione (GSH) in 100 mM

233    sodium phosphate buffer (pH 6.5) immediately prior to being mixed with the supernatant. A pH

234    of 6.5 was used for the substrate mixture to minimize auto conjugation of GSH with CDNB. 180

235    μL of substrate mixture was added to each well of supernatant for a final concentration of 0.9

236    mM CDNB and 0.86 mM GSH. The plate was read at 340 nm in 1 min intervals for 5 min. An

237    experimentally derived extinction coefficient of $0.00580\ \mu M^{-1}\ cm^{-1}$ was used that accounted for

238    the path length through the 200 μL total volume in each well.

239        Total protein was measured with the Bradford method using bovine serum albumin as a

240    standard ($R^2 = 0.978$) [25]. Units for all four enzyme assays were MFO content as μg

241    cytochrome c equivalents/mg protein, and CarE and GST specific activities as nmol

242    substrate/min/mg protein.

243

## Statistical analyses

245        All analyses were conducted in R version 4.1.1 [26].  Enzyme activities were compared

246    by Kruskal-Wallis tests due to this test being robust to smaller sample sizes. When applicable, a

247    Mann-Whitney-U test was used for pairwise comparisons between the strains. Two analyses

248  were done, one that included DPN1 and another that included DPN2. This was done to simplify

249  the analyses because DPN1 and DPN2 are repeated measures of the same strain, thus not

250  independent, and all other strains were independent. Each enzyme activity assay also was

251  regressed against percent mortality at both 30 min and at 120 min and the slopes were used to

252  determine an effect of enzyme activity on percent mortality. This was done by building general

253  linear models, which were visually assessed for heteroscedasticity and normality of residuals.

254      For CDC bottle bioassays, a clustered Cox regression was generated on pairwise

255  comparisons of Sumithrin® and Anvil® or Sumithrin® and AnvilTM at the 30 min diagnostic

256  time point used by the CDC to assess resistance [19] using the 'survival' package [16]. The

257  clustering effect was assigned to the multiple bottles for each of the treatments. Prior to statistical

258  testing, the Cox models were assessed for proportionality using the 'cox.zph()' function. Models

259  that violated the assumption of proportionality had a time-dependent coefficient added to them

260  (i.e., an interaction term between time in minutes and treatment), and these hazard ratios are

261  reported with a time factor change in hazard rate of mortality. For ties in times to death, the

262  Efron approximation was used. A Wald test was used to test the null hypothesis that the beta

263  coefficients = 0 at $\alpha = 0.05$ and results are reported as fold change in the hazard rate of mortality

264  in the Anvil® or AnvilTM (both Sumithrin® + PBO) treatment compared to the Sumithrin®

265  treatment. Bottle bioassays were analyzed only if at least three replicates of each treatment were

266  done per location. Thus, analyses of AHB, WHE, and DPN were included.

267

## Results

## Bottle bioassays

270      The distribution of observed mortality over 30 min differed in several strains when

271 comparing Sumithrin® technical (20 µg/bottle) against either Anvil® at 22.17 µg Sumithrin®

272 and 22.17 µg PBO or an Anvil® tank mix (AnvilTM) equivalent with 11.09 µg Sumithrin® and

273 11.09 µg PBO in 1:1 mineral oil (Table 3). Mortality reached 100% in the Anvil® treatment by

274 the diagnostic time of 30 min but was 70% against the technical Sumithrin®. There was a

275 significant difference between Sumithrin® and Anvil® in AHB mosquitoes (Fig 3; HR = 3.85

276 (2.496 – 5.949 95% CI), $P < 0.001$). There was a 3.9-fold change in hazard rate of mortality

277 when mosquitoes were exposed to the Anvil® treatment over the course of 30 min.

278

279 **Table 3. Percent mortality of five different strains of *Culex pipiens* complex when exposed**

280 **to a diagnostic dose of Sumithrin® (20 µg/bottle), Anvil® (22.2 µg/bottle Sumithrin® + 22.2**

281 **µg/bottle PBO), or AnvilTM (11.1 µg/bottle Sumithrin® + 11.1 µg/bottle PBO cut 1:1 with**

282 **mineral oil) from onset of treatment to the diagnostic time point (30 min) and the study end**

283 **point (120 min).**

| | % Mortality[1] | | | | | |
|---|---|---|---|---|---|---|
| | After 30 min | | | After 120 min | | |
| Strain | Anvil® | AnvilTM | Sumithrin® | Anvil® | AnvilTM | Sumithrin® |
| AHB | 100 | - | 70.0 | 100 | - | 95.7 |
| WHE | 100 | 88.8 | 51.4 | 100 | 97.2 | 86.5 |
| DPS | - | - | 10.5 | - | - | 68.4 |
| DPSB | 88.2 | 100 | 82.6 | 100 | 100 | 100 |
| DPN | 82.4 | - | 72.1 | 98.8 | - | 91.8 |
| DPN1 | 97.8 | 98.5 | 76.6 | 100 | 100 | 100 |

| DPN2 | - | - | 61.1 | - | - | 100 |

284     [1] CDC guidelines consider samples with a mortality at the diagnostic time of $> 97\%$ are

285     susceptible, $96 - 90\%$ are building resistance, and $< 90\%$ are resistant [19].

286

287     **Fig 3. Proportion mortality/survival of Arlington Heights catch basin (AHB) adult *Culex***

288     ***pipiens* complex in CDC bottle bioassays, with either Sumithrin or Anvil, after 120 minutes**

289     **of continuous exposure.** Top panel: common mortality curve. Bottom panel: Kaplan-Meier

290     survival curves and 95% confidence intervals (shaded area). Vertical dotted line at 30 minutes

291     denotes the diagnostic time for Sumithrin®.

292

293     Mortality also reached 100% in Anvil® at the diagnostic time in WHE. The hazard ratio

294     was not proportional over time by a factor of 0.91 (Fig 4; HR = 0.91 $(0.841 - 0.992)$, P = 0.032),

295     and there was a significant difference between Sumithrin® and Anvil® (HR = 36.11 $(8.465 -$

296     154.007 95% CI), P $< 0.001$). Thus, there was a 36.1-fold change in hazard risk of mortality in

297     the Anvil® treatment at zero min that changed by 0.91 every minute (i.e., 36.1 x $0.91^n$, where n

298     = minutes). At 30 min, the hazard rate of mortality in the Anvil® treatment was about a 2.1-fold

299     compared to Sumithrin® alone. The hazard also was not proportional over time between

300     Sumithrin® and AnvilTM at 30 min (HR = 0.91 $(0.858 - 0.970)$, P = 0.003). The hazard ratio

301     significantly differed between Sumithrin® and AnvilTM (HR = 17.33 $(4.707 - 63.817)$, P $<$

302     0.001). There was a 17.3-fold change in hazard rate of mortality in the AnvilTM treatment

303     starting at zero min and changed by 0.91 every minute for 30 min. By 30 min, the hazard rate of

304     mortality was about double in the Anvil® treatment.

305

306 **Fig 4. Proportion mortality/survival of Wheeling (WHE) adult *Culex pipiens* complex in**

307 **CDC bottle bioassays, with either Sumithrin, AnvilTM, or Anvil, after 120 minutes of**

308 **continuous exposure.** Top panel: common mortality curve. Bottom panel: Kaplan-Meier

309 survival curves and 95% confidence intervals (shaded area). Vertical dotted line at 30 minutes

310 denotes the diagnostic time for Sumithrin.

311

312       There was no difference in hazard rate of mortality between Sumithrin® and Anvil® in

313 the DPN samples (Fig 5; HR = 1.26 (0.605 – 2.629), P = 0.537).

314

315 **Fig 5. Proportion mortality/survival of Des Plaines North (DPN) adult *Culex pipiens***

316 **complex in CDC bottle bioassays, with either Sumithrin or Anvil, after 120 minutes of**

317 **continuous exposure.** Top panel: common mortality curve. Bottom panel: Kaplan-Meier

318 survival curves and 95% confidence intervals (shaded area). Vertical dotted line at 30 minutes

319 denotes the diagnostic time for Sumithrin.

320

321

## Genotyping for knockdown resistance alleles

323       All five strains tested were positive to varying degrees for the 1014F allele (Table 4). The

324 DPS strain had the highest percent homozygosity for the *kdr* genotype (FF), as well as the

325 highest frequency for the 1014F allele. No homozygotes for the susceptible genotype (LL) were

326 identified among the tested DPS organisms. The DPN strain that was genotyped was from a

327 collection earlier than those used in the bottle bioassay and enzyme activity assays and had the

328 lowest percent homozygosity of the FF genotype, as well as the lowest 1014F allele frequency.

329    Heterozygosity (genotype LF) was generally high compared to both homozygous genotypes in

330    all five strains.

331

332    **Table 4. Percent genotype and allele frequency of the leucine-to-phenylalanine (L1014F)**

333    **knockdown resistance mutation in the voltage-sensitive sodium channel of adult female**

334    ***Culex pipiens* complex from some Northwest suburbs of Chicago, Illinois.**

|  |  |  |  |  | Allele Frequency | |
| --- | --- | --- | --- | --- | --- | --- |
|  |  | Percent Genotype | | |  | |
| Strain | n | FF | LF | LL | F | L |
| AHB | 44 | 25.0 | 68.2 | 6.8 | 0.59 | 0.41 |
| WHE | 46 | 41.3 | 54.3 | 4.3 | 0.68 | 0.32 |
| DPS | 37 | 43.2 | 56.8 | 0 | 0.72 | 0.28 |
| DPSB | 38 | 18.4 | 65.8 | 15.8 | 0.51 | 0.49 |
| DPN | 43 | 9.3 | 76.7 | 14.0 | 0.48 | 0.52 |

335

## Enzyme activity assays

337        The analysis was first done with all strains including DPN1 but not DPN2 and then all

338    strains including DPN2 but not DPN1. Finally, DPN1 and DPN2 were compared. This was done

339    because DPN1 and DPN2 were repeated measures of the DPN strain and thus not independent.

340    Activity of GSTs were only significantly different when DPN2 was included in the group (Fig 6;

341    $\chi^2 = 10.59$, df = 3, P = 0.014). AHB was significantly different from DPS (W = 54, P = 0.021)

342    and DPN2 (W = 55, P = 0.015) and WHE was significantly different from DPN2 (W = 10, P =

343    0.021). No other enzyme activities or MFO content were significantly different among the

344  strains. There also was no difference in any of the enzyme activities or MFO content between

345  DPN1 and DPN2 (all P > 0.130). The coefficient of variation for $\alpha$-CarE and $\beta$-CarE in the AHB

346  strain were the two highest among all tested strains and enzyme activities (Table 5). There was

347  no effect of enzyme activity or MFO content that explained the differences in percent mortality

348  at either 30 min or at 120 min (i.e., all regression slopes were statistically equal to zero).

349

350  **Fig 6. Distributions of specific activities for mixed-function oxidase (MFO), glutathione *S*-**

351  **transferase (GST), alpha-carboxylesterase ($\alpha$-CarE), and beta-carboxylesterase ($\beta$-CarE)**

352  **in female *Culex pipiens* complex from four different trapping locations in some Northwest**

353  **suburbs of Chicago, Illinois.** The lower and upper hinge of boxes represent the 25th and 75th

354  quartiles, respectively. The middle line represents the median. The whiskers represent the

355  minimum and maximum range of the data.

356

357  **Table 5. Coefficient of variation for mixed-function oxidase (MFO), glutathione *S*-**

358  **transferase (GST), alpha-carboxylesterase ($\alpha$-CarE), and beta-carboxylesterase ($\beta$-CarE)**

359  **specific activities in female *Culex pipiens* complex from four different trapping locations in**

360  **some Northwest suburbs of Chicago, Illinois, USA. Larger numbers indicate greater**

361  **variability relative to their mean.**

| Strain | *MFO* | *GST* | *α-CarE* | *β-CarE* |
|--------|-------|-------|----------|----------|
| AHB    | 23.6  | 19.6  | 76.5     | 87.3     |
| WHE    | 22.7  | 28.0  | 23.1     | 29.0     |
| DPS    | 30.6  | 25.6  | 25.6     | 23.7     |
| DPN1   | 13.9  | 24.6  | 34.2     | 35.2     |

DPN2    22.2    28.4    28.3    33.1

362

# Discussion

364        According to CDC guidelines [19], all five strains in the present study may be resistant to

365    Sumithrin®, and the formulated product Anvil® reclaimed efficacy in three out of five strains.

366    This is not surprising given the frequency of ULV applications in these areas during the time the

367    mosquito collections were made. Previous studies have demonstrated resistance in both adult

368    [27] and larval [28, 29] *Cx. pipiens* in the suburbs of Chicago. With historically high incidence

369    of WNV in the Chicago area when compared to the rest of the upper Midwest and northeast US

370    where *Culex pipiens* and *Culex restuans* are the main vector. [30], monitoring resistance status in

371    *Cx. pipiens* is of public health importance. Knowing what mechanisms these strains rely on to

372    survive chemical control will help to inform mosquito abatement districts on application timing

373    and product rotation.

374        Numerous resistance mechanisms have been implicated in *Cx. pipiens* (reviewed in [15]).

375    Although *Culex* spp. are known to have a leucine-to-phenylalanine or leucine-to-serine single

376    nucleotide polymorphism (SNP) at the $982^{nd}$ amino acid in the para region of the voltage-

377    sensitive sodium channel (structurally the same location as the L1014F/H mutation in house

378    flies), their roles in resistance to pyrethroids is acknowledged but not well-resolved (e.g., [31,

379    32]). In the case of the leucine-to-serine mutation, there appears to be stronger resistance to DDT

380    than to pyrethroids [33]. The house fly L1014F mutation is only known to confer moderate levels

381    of resistance by itself [34] compared to bi- and tri-allelic *kdr* combinations [35]. In the case of

382    the *Cx. pipiens* complex, hybridization rate may also play a role in expression of *kdr* [1]. In the

383    present study, *kdr* alone does not appear to explain the relatively large differences in mortality to

384    Sumithrin® at 30 and 120 min but does appear to have some involvement. The DPS strain had

385    the lowest mortality (10.5%) at the diagnostic time of 30 min and had the greatest resistant

386    phenylalanine (F) allele frequency, but WHE had similar allele frequency and resulted in over

387    40% greater mortality (51.4%) over 30 min. WHE had 100% mortality with Anvil® treatment,

388    suggesting significant involvement of MFOs and/or CarEs.

389        Tests utilizing synergists in the CDC bottle bioassay can suggest enzymatic

390    detoxification playing a role in resistance [36, 37], especially in the presence of high L982F *kdr*

391    allele frequencies [38]. That we saw considerable restoration of susceptibility when these strains

392    were tested with Anvil®, a Sumithrin® product that is synergized with PBO, is consistent with

393    previous findings (e.g., [38]). Traditionally, PBO is thought to inhibit cytochrome P450s (aka

394    MFOs), a key enzyme in many oxidative processes, including insecticide metabolism ([39]). But

395    PBO also has been shown to inhibit carboxylesterases [40]. A correlative relationship with

396    enzyme activity and resistance to deltamethrin, permethrin, and DDT in *Cx. quinquefasciatus*, a

397    close relative to *Cx. pipiens*, has been previously shown [41]. Xu et al. [42] found over 1,000-

398    fold difference in $LC_{50}$ in two resistant strains of *Cx. quinquefasciatus* that were selected with

399    permethrin. PBO contributed significant reduction in $LC_{50}$ values, down to around 100-fold, of

400    the resistant strains compared to the susceptible strain. Although methodology is different in the

401    present study, the results of the bottle bioassays are consistent with PBO contributing significant

402    restoration of Sumithrin® efficacy, suggesting that either cytochrome P450s and/or

403    carboxylesterases play a role in the resistance phenotype among these strains. Similar restoration

404    of susceptibility was recently demonstrated in populations of *Cx. quinquefasciatus* from Florida

405    and California [36, 37].

406    In the case of the MFO activity assay, 3',3,5',5'-TMB is a substrate used to measure

407    heme content in a sample, of which most is thought to be attributed to cytochrome P450s [23].

408    This assay is considered a surrogate assay and does not measure activity directly as in other types

409    of activity assays, such as the CarE and GST assays in the present study. Although regressions of

410    bottle bioassay mortality did not suggest a relationship based on MFO quantity, CarE, or GST

411    activities in the control mosquitoes, it does not rule out altered enzyme activity toward

412    Sumithrin®. It also does not rule out inducible overexpression of these enzymes, especially

413    MFOs, which have been documented in resistant *Cx. quinquefasciatus* [43]. This is a plausible

414    explanation for the discrepancy between enzyme activities and PBO synergism because

415    induction usually happens shortly after initiation of exposure to a toxicant, and enzyme activities

416    conducted in the present study were done on control mosquitoes (i.e., not exposed to

417    Sumithrin®). Regardless of the dynamics of cytochrome P450 expression, PBO tends to reduce

418    pyrethroid $LD_{50}$s even in lab-reared, fully susceptible *Culex* spp. (e.g., [37]), and *Musca*

419    *domestica* (e.g., [44]).

420    Although limited in what they can tell us about enzymatic involvement in resistance, an

421    application of enzyme activity assays that may be useful in describing the dynamics of the

422    enzymatic role in resistance is in an induction experiment. In an induction experiment, the

423    researcher exposes the animals to a sublethal dose of the insecticide and collects individuals over

424    a time series. The researcher then runs activity assays on the specimens and relates the activities

425    back to toxicological response data. The assay in the present study only had the potential to

426    detect constitutively expressed detoxification enzymes. The goal of a future study in *Cx. pipiens*

427    complex adults from the Chicago area should include an induction experiment with Sumithrin®.

428    That the synergized formulation of Sumithrin® and PBO in Anvil® restored susceptibility in

429     multiple strains suggests that expression of MFOs and/or CarEs may be induced at high levels

430     upon exposure to a toxicant like Sumithrin®, or perhaps even have a target site mutation that

431     confers an advantage in processing Sumithrin®. Another explanation could be that the model

432     substrates used in the present study simply do not interact with the isozymes that may play a role

433     in *Cx. pipiens* complex resistance to Sumithrin®. This could also be true of any induction

434     experiment undertaken in future studies.

435        We note that in the AHB strain, there was a high coefficient of variation in the CarE

436     activities relative to the other strains. The inclusion of PBO with Sumithrin® appeared to

437     provide satisfactory control in this strain at the time of data collection and should be considered

438     in future regional control efforts. WHE had notable resistance to Sumithrin® but similarly the

439     addition of PBO in Anvil® provided 100% mortality at the diagnostic time of 30 min. AHB and

440     WHE had the greatest difference in mortality at 30 min diagnostic period between Sumithrin®

441     and Anvil® (30% and 48.6%, respectively). The DPS strain showed the highest resistance

442     according to CDC diagnostic dose and time for Sumithrin®. DPS also showed the highest F

443     allele frequency and the highest percentage of resistant FF homozygotes. DPS was not screened

444     with Anvil® and there was no indication of enzymatic involvement based on the activity assays.

445     A follow up study should focus on the DPS strain and include an Anvil® bottle bioassay to

446     measure an effect, if any, that MFOs and/or CarEs have on its resistance. Interestingly, neither

447     DPSB nor two of the DPN resamples reverted to 100% mortality when treated with Anvil®. By

448     the second resampling (DPN2), resistance to Sumithrin® increased by 11%.

449        Examined from the perspective of hazard ratios, in the case of AHB and WHE, we saw

450     that treatment with Anvil® resulted in a 3.9-fold and 2.1-fold change in the risk of mortality at

451     30 min compared to Sumithrin®. In DPN, the raw bottle bioassay mortality data shows only a

452    small increase in mortality in the Anvil® treatment, which statistically conferred no greater risk

453    of mortality than the Sumithrin® treatment. With the inability to calculate informative numbers

454    such as resistance and synergist ratios (e.g., [45, 46]) using diagnostic doses in bottle bioassays,

455    hazard ratios from Cox regression offer the potential to make ratio-based quantitative

456    comparisons among treatments. In the present study, the choice to analyze bottle bioassay data

457    using Cox regression was made after bottle bioassays had already been run and some potentially

458    important factors such as sex and genotypes were not collected. The inclusion of these types of

459    factors would help to explain results like these in future studies. We recommend the use of

460    clustered Cox regression with time-dependent covariates when describing how much risk

461    multiple variables contribute to the rate of mortality across a given diagnostic time period.

462    Typical analyses of bottle bioassays, including binomial generalized linear models and repeated

463    measures ANOVA, violate several important assumptions of these statistical tests, including the

464    independence of observations [47], which seldom allow for the correct grouping variables to

465    account for this fact. This can be accounted for using a clustering effect in Cox regression, which

466    is akin to a random factor in a mixed model. Quite often, studies that utilize Kaplan-Meier

467    survival analysis are not assessed for the assumption of proportional hazards, a key assumption

468    that must be met for model estimates to be deemed accurate [16]. This is evident by the often-

469    seen crossing of survival curves in a figure (e.g., Fig. 4). We have provided code for readers to

470    use on future analyses of bottle bioassay data using Cox regression. Cox regression is a benefit to

471    the interpretation of bottle bioassay data because it allows multiple factors to be assessed

472    simultaneously across numerous time points, not just a single endpoint. We liken these two

473    statistical comparisons to taking a picture (end-point analysis) versus watching a movie (Cox

474    regression analysis). For instance, in Figure 3 (WHB) mortality in the Anvil® and AnvilTM

475 treatments were in excess of 75% within the first 10 and 15 min, respectively, at which point an

476 inflection in the rate of mortality can be seen. With downstream molecular and biochemical

477 analyses, this point of inflection could be characterized, with Cox regression as the statistical

478 method to infer risk of mortality due to those types of factors. Future studies on *Cx. pipiens*

479 resistance in field strains, where topical application of pesticides is not feasible, should

480 incorporate this type of analysis to make clearer distinctions among the numerous resistance

481 mechanisms and their relative impact on operational success.

482

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493

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495

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